Published in final edited form as:
Nat Rev Microbiol. 2009 May ; 7(5): 383–392. doi:10.1038/nrmicro2056.

Using Movies to Analyse Gene Circuit Dynamics in Single Cells

James CW Locke[1] and **Michael B Elowitz**[1]
[1]Howard Hughes Medical Institute, Division of Biology and Department of Applied Physics, California Institute of Technology, Pasadena, California 91125, USA

Preface

Many bacterial systems rely on dynamic genetic circuits to control critical processes. A major goal of systems biology is to understand these behaviours in terms of individual genes and their interactions. However, traditional techniques based on population averages wash out critical dynamics that are either unsynchronized between cells or driven by fluctuations, or 'noise,' in cellular components. Recently, the combination of time-lapse microscopy, quantitative image analysis, and fluorescent protein reporters has enabled direct observation of multiple cellular components over time in individual cells. In conjunction with mathematical modelling, these techniques are now providing powerful insights into genetic circuit behaviour in diverse microbial systems.

As biologists we must grapple with, and reconcile, two very different views of cellular behaviour: On the one hand, we frequently think of cellular functions as being determined by 'circuits' of interacting genes and proteins. These chemical circuits, loosely analogous to electronic circuits, encode genetic programs that underlie differentiation, the cell cycle, and other behaviours (Fig 1a). They accurately respond to stimuli and generate precise behavioural programs in individual cells. On the other hand, there is the 'noisy' view of the cell we get whenever we actually look at cells: squishy, dynamic, and heterogeneous populations whose morphologies, gene expression patterns, and differentiated states differ from one another, even when environment and genotype are fixed (Fig1b). How can precisely defined genetic circuits give rise to heterogeneity and, conversely, how does heterogeneity affect the behaviour of circuits?

Movies offer a powerful way to address these questions (Fig 1c). By engineering microbial strains to express fluorescent protein reporters for key genes one can follow the changing characteristics of individual cells over time. Quantitative detection methods, improved microscope automation and software, and the variety of fluorescent reporter genes, in conjunction with mathematical modelling, can be combined to analyse gene circuit dynamics. Together, these techniques allow researchers to characterize epigenetic states, identify new dynamic phenomena, analyse biochemical interactions within circuits, and elucidate the physiological function of genetic circuits, all at the single-cell level. Finally, movies provide an aesthetically compelling view of cellular function that is often fascinating to watch. We have found that the eye often picks up on subtle patterns in individual living cells that would be difficult to notice with less direct techniques. Few techniques are more fun.

How does quantitative movie analysis compare to alternative techniques for analyzing gene circuits? Time-lapse microscopy follows a few genes over time in individual living cells. It complements approaches such as microarrays (which provide genome-scale expression data averaged over populations but do not allow analysis of variability) and flow cytometry (which allows high throughput acquisition of single cell fluorescence values but does not allow the same cells to be tracked over time). Movies also complement new single-cell Q-PCR approaches, which enable analysis of expression of multiple genes in individual cells, but, because they require lysis of the cell, do not permit tracking of expression dynamics[1]. Movies

alone permit one to determine the "trajectories" of gene expression levels in individual living cells. One potential drawback of movies, is that, for a particular process under study, many genes, and their expression levels, may be important, but most studies presently follow the dynamics of only a few genes at a time due to the lack of distinguishable reporters. In the future, multi-spectral techniques may expand the number of simultaneous reporters[2]. However, following the dynamics of only 2 or 3 genes at once can still be extremely informative.

Here we will review work in which movies provide new insights into the dynamic behaviour of genetic components and circuits. For this review, we have confined ourselves to microbial systems, and have therefore excluded much interesting recent work in mammalian cell culture and multicellular organisms [3-5]. Because our focus is on gene circuit dynamics we have also excluded a growing literature of fascinating studies on the sub-cellular localization dynamics of individual cellular components [6-11]. We will first review recent work where time-lapse microscopy has been used to examine and characterize variability in single cell gene expression. Next, we describe how movies can facilitate quantitative analysis of biochemical interactions in individual cells. Finally, we explore how movies can provide an integrated picture of genetic circuit dynamics and thereby reveal key principles of genetic circuit design. Taken together, these studies are beginning to reveal intimate connections between the deterministic "circuit" and heterogeneous "noisy" views of the cell described above: On the one hand, genetic circuits crucially generate and control fluctuations in cellular components and heterogeneity in cellular states. Conversely, variability is essential for at least some genetic circuits to function properly.

The origins of variability

If all cells behaved the same, it would be unnecessary to analyse individual cells. Measurements showed that gene expression is often quite variable, even in clonal cell populations grown in identical environments [12-14] Where does that variability originate? It can arise from stochastic fluctuations, or 'noise,' in cellular components and biochemical reactions [12,15]. These fluctuations are expected to be significant given the low copy numbers of key molecular species in the cell[16,17]. However, variability can also reflect differences in the micro-environments inhabited by individual cells. Furthermore, pre-existing heterogeneity can be propagated to subsequent cell generations. Such effects can be observed by following lineages over several cell-generations. The interplay between these sources of variability is addressed in several recent studies using movies.

Lineage analysis allows tracking of epigenetic states

In the 1950s and 1960s, E.O. Powell and others used phase contrast microscopy to observe the growth of bacterial micro-colonies [18,19]. They carefully analysed the heritability of growth rates and other phenotypes, and suggested that stochastic fluctuations in cellular components might generate the observed variability. Improved acquisition techniques (Box 1, Fig 2) now enable researchers to address these questions more systematically. Rather than tracking colonies for 2-3 generations by eye, it is now possible to record growth automatically over many generations[20]. Perhaps the best example of how lineage can affect cell-cell variability, observed in diverse multicellular systems and in yeast [21], comes from the study of aging cells. Symmetrically dividing bacteria, such as *E. coli*, have no obvious means of aging. However, by tracking over 35,000 cells using an automatic tracking program, and using a flat microcolony growth protocol (Box 2) Stewart et al. showed that *Escherichia coli* indeed age[22]. After division, each daughter cell has one new pole, created by the septation event, and one old pole inherited from the parent. By sorting the lineage tree by pole age, one can observe a systematic reduction in growth rate for cells with more older poles, as well as an increased chance of death (Fig. 3A). Without a specific molecular marker for cell age, it is difficult to imagine how such effects could have been observed using traditional techniques.

Persistence at the single-cell level

When antibiotics are added transiently to a population of bacterial cells, most of the population is killed. In some cases, however, a small percentage of "persister" cells survive antibiotic treatments, grow, and re-establish the population [23]. A second pulse of antibiotics leads to a similar result, showing that persistence is not due to mutation [24]. But is persistence induced by the antibiotic, or do some cells spontaneously enter a persistent state before the addition of antibiotics? The persister state can be identified in movies because it causes strongly reduced cell growth. Using time-lapse microscopy of cells embedded in linear micro-fluidic chambers, Balaban et al showed that individual *E. coli* cells switch in and out of the persister state spontaneously, in the absence of antibiotics [25]. In a second study, the group used movies to identify an intermediate state in the transition to persistence during which cells stop growing but continue to express proteins[26]. Cells remain susceptible to antibiotics during this period.

This methodology could provide insight into biomedically relevant pathogens, including the slow-growing mycobacteria responsible for tuberculosis [23,27]. Being able to study rare spontaneous state changes may help identify strategies that influence the susceptibility of persistent infections to drug treatments. Currently a limitation of this approach is the difficulty in analyzing extremely rare events which become increasingly difficult to find at the very low frequencies that characterize some natural persister states (e.g. 10^{-6} for *E. coli*). To circumvent this problem, the authors used previously identified mutants which exhibit an elevated frequency of persisters[28]. With these imaging techniques established an important challenge is now to work out the underlying circuit responsible for inducing state changes in a probabilistic fashion. In particular, it will be interesting to see whether this circuitry is similar to the excitable genetic circuit responsible for competence induction in *B. subtilis*, described below.

Heritability of cellular states

Cell state heterogeneity can be analysed without movies, but movies can provide additional insights into the process. For example, Acar et al recently analysed the galactose utilization system in yeast. They showed that yeast cells mutant for one feedback loop appear to spontaneously switch between states of high and low expression of galactose utilization genes. But it remained unclear whether (and how) these states are inherited across cell generations (division events). Kaufman et al. addressed this issue by examining the heritability of such gene expression states [29] by tracking yeast cells over 15 hours through ~6 divisions in movies (Fig 3B-D) Remarkably, mother and daughter cells switch on the GAL pathway synchronously after division, indicating that the timing of these apparently random decisions is heritable. The authors explain this behaviour in terms of a model based on a single fluctuating regulatory protein that is synthesized in large bursts. Clearly, the interplay between stochastic switches and heritable states can be complex.

Cell-cycle variability

As an unsynchronized dynamic oscillatory process continuously operating in individual cells, the cell cycle represents a key potential source of variability, but how variable is the cell cycle itself? And where does that variability originate? When examined at the single-cell level cell-cycle progression can be strikingly variable. Movies have been used to quantify variability in the timing of specific cell-cycle stages in yeast [30,31]. This work broke overall variability in timing into steps that were either dependent or independent of cell size. The first step is responsible for controlling cell size prior to division. Interestingly, variability in the cell-size-independent time interval was reduced by increasing ploidy in a manner consistent with stochastic variation in expression of certain genes, such as G1 cyclins.

In a complementary study, Ramanathan and co-workers dissected timing variability leading up to the cell-fate decision in yeast to initiate meiotic sporulation[32]. This study showed that the timing of sporulation varies considerably among cells, even though all cells activate expression of the master regulator of sporulation at roughly the same time. Variability in the decision to initiate sporulation results in part from slow and variable accumulation of the master regulator Ime1p.

These studies have begun to examine molecular mechanisms by which cell cycle progression can be intrinsically noisy. It will be interesting to now see how this variability impacts other cellular processes, and to what extent this variability is adaptive.

Movies of clocks

In contrast to the inherently variable oscillations of the cell cycle, circadian clocks are remarkable for their temporal precision. Cyanobacteria possess a 24-hour circadian clock, whose molecular components have been identified and shown to reconstitute oscillations in vitro [33,34]. Although colonies and cultures were known to exhibit robust rhythms, it was unclear how accurately the clock performed at the single-cell level, given the possibility of substantial noise in the levels of its molecular components. The possibility that the circadian clock might be inherently noisy was suggested by studies of an unrelated synthetic genetic clock, termed the Repressilator. Using movies, it was shown that the repressilator generates robust self-sustaining (limit cycle) oscillations in *E. coli* cells [35]. However, the clock is erratic and its state quickly becomes desynchronized within the population. In contrast, when Mihalcescu et al used a sensitive luciferase reporter system to image clock state in individual cells of growing microcolonies over time [36,37], they found that individual cells oscillated with a robust 24h rhythm. When two microcolonies with different clock phases were placed next to each other on the same pad, they did not influence each other's phase, suggesting that clock accuracy was cell-autonomous [36,38] and did not require active synchronization [39]. A mechanism for the cyanobacterial clock circuit based on interactions among three key proteins KAI A,B and C has recently been established [34,40]. The post-translational nature of this clock circuit mechanism may help to explain its apparent robustness to noise.

Together these studies show a range of examples in which variability is generated, inherited, or suppressed in different circuits. They thus raise the question of how the circuit level variability relates to specific biochemical interactions in the cell.

In vivo biochemistry

Surprisingly, movies can provide the type of detailed and quantitative analyses of biochemical interactions that are traditionally determined using gels, blots and other biochemical assays. Several recent studies push the limit on quantitative analysis of movie data.

The Gene Regulation Function

One of the most basic characteristics of a genetic circuit is the interaction between a transcription factor and its target genes. This interaction can be summarized by the effective gene-regulation function (GRF): the relationship between the concentration of one or more transcription factors in a cell, and the rate of production of its target gene. The shape of the GRF is critical for the function of gene circuits. However, population-average measurements can “smear out” these response functions, and *in vitro* measurements may not reflect the many effects of the intracellular environment. Thus, GRFs must be measured in individual living cells.

In order to measure a typical GRF, Rosenfeld et al engineered a strain of *E. coli* in which a lambda repressor-YFP protein repressed the expression of a cyan fluorescent protein (CFP)

from one of its key target promoters. They allowed the tagged repressor to dilute out as the cells grew into microcolonies, while simultaneously monitoring the rate of increase of CFP fluorescence (Fig 4A-B, Supplementary Information S1 (Movie)). These data give the GRF for the lambda repressor-promoter interaction. Critically, they showed that the GRF was not a well-defined function. Rather, CFP expression fluctuates slowly, with a typical timescale of one cell cycle. Thus, the output of the repressor-promoter system is not solely determined by the concentration of repressor in the cell. Because these fluctuations are slow, the cell would require several generations to "average them out". In this way, GRF fluctuations fundamentally limit the accuracy of transcriptional regulation.

The measured GRF had remarkably accurate predictive value: when synthetic negative-feedback circuits were constructed using only the measured promoter, they exhibited the mean expression levels expected from a simple genetic circuit model, with no free parameters[41]. Thus, these techniques will likely facilitate quantitative synthetic biology approaches[42-44]. A similar example in which movies have been used to characterize biochemical systems useful for synthetic biology was recently taken by Grilly et al, who characterized a prokaryotic protein-degradation system in yeast. In order to determine the protein-degradation rates, they simply tracked the fluorescence of a GFP construct tagged for degradation in single cells [45].

Single molecules: the final biochemical frontier—Perhaps the ultimate limit to *in vivo* analysis of biochemical interactions is detection of individual molecules within a cell. Recently this milestone was reached in a pair of pioneering studies in *E. coli* from S. Xie and colleagues[46,47]. First, Yu et al fused YFP to a membrane protein to reduce its mobility in the cell sufficiently that the integrated fluorescence from each molecule could be detected above the background autofluorescence of the cell (Fig.4c). Second, Cai et al measured expression of individual molecules of the well-characterized enzyme beta-galactosidase, observing quantized rates of production of a fluorescent substrate in a microfluidic chamber containing a single cell. The ability to track the production and degradation of individual molecules over time using movies enabled development of a predictive model of the distribution of protein numbers across cells and generated evidence for, and characterization of, burst-like protein expression [48].

Together these studies and others like them will help bridge the gap between the biochemical interactions between individual molecules, as they occur in cells, and the behaviour of more complex circuits consisting of multiple components and interactions.

Movies reveal circuit dynamics

A variety of techniques have begun to reveal the structure of genetic circuits: who regulates whom, and how. However, despite this information it remains difficult to predict the cellular dynamics that those circuits will generate. This is especially true for circuits that are only active in a sub-population of cells, or whose behaviour is highly variable. In *B. subtilis* under stress, a small percentage of cells (5–10%) enter a state of competence where they can readily take up exogenous DNA [49,50]. Similarly, *B. subtilis* undergoes a dramatic differentiation process in which a cell transforms into a dormant spore. Individual cells vary significantly in when, and in some cases whether, they initiate sporulation. What accounts for this variability in cell fate decision-making? Recently, movies have allowed researchers to connect the decision-making behaviours of individual cells to the architecture of underlying genetic circuits in both competence [51,52] and sporulation[53].

Transient, probabilistic differentiation

Pioneering work by D. Dubnau, P. Zuber, A. Grossman, and others established the key molecular interactions necessary for competence (Reviewed in [54]). The transcription factor

ComK is both necessary and sufficient to induce cells into the competent state, and positively autoregulates its own expression. Expression of ComS is necessary, but not sufficient, for inducing competence. In movies cells appear to spontaneously activate ComK and, after some time, revert back to vegetative growth. What accounts for the probabilistic, unsynchronized, activation of competence in only a fraction of cells? And, how do cells ensure a timely exit from the competent state?

Movies of *B.subtilis* cells containing reporters for pairs of genes during entry and exit from competence provided clues to address these questions. For example, cells exhibited a strong anti-correlation between ComK and ComS: as soon as ComK levels switched 'on', ComS expression switched 'off', and vice versa (Fig. 5A-C, Supplementary Information 2 (movie)), consistent with ComK directly or indirectly repressing the expression of ComS to form a negative-feedback loop. When combined with positive autoregulation of ComK, the two feedback loops together explain the probabilistic and transient nature of competence in terms of excitable dynamics that are mathematically similar to action potentials in neurons [55]. But what is the 'trigger' for differentiation? In the mathematical model of competence based on excitability, noise –fluctuations in molecular components like ComK – triggers differentiation. To test this idea, cells mutant for the septation gene ftsW were used. These cells grew into long filaments. Because of their increased size, they exhibited reduced noise (fluctuations in gene expression 'averaged out' more in the larger cells), but similar mean levels of gene expression. They also exhibited progressively reduced propensity to differentiate at longer lengths, supporting the idea that fluctuations are necessary for differentiation in this system[52].

D. Dubnau and colleagues approached this question from a complementary and more direct point of view: They tested the hypothesis that fluctuations in ComK expression were responsible for initiating differentiation. They generated strains differing in the noisiness, but not the mean rate, of ComK expression. By reducing the rate of ComK translation while increasing its rate of transcription, the expression of ComK was made less "bursty." Strikingly, the frequency of differentiation was reduced[56]. These experiments established that ComK noise affects differentiation propensity. This approach could be extended to test the role of noise in other factors as well, in competence as well as other systems.[50,56].

Sporulation: terminal differentiation

In *B. subtilis*, entry into the sporulation pathway is controlled by the master transcription regulator Spo0A, whose expression is heterogeneous[57,58]. Veening et al used time-lapse movies of a strain with a fluorescent spo0A reporter gene to analyse the decision of individual cells to sporulate (or not) (Fig 5D). Their movies revealed that *B. subtilis* implements a 'bet hedging' strategy whereby some cells sporulate (high Spo0A activity) while others use alternative metabolites to continue growing (low Spo0A activity). By analyzing cell lineages, they showed that variations in the propensity to sporulate persist up to 2 generations. Interestingly, these results are similar to those seen for the inheritance of states in a bistable switch in yeast [29].

Spo0A becomes transcriptionally active upon phosphorylation by a multi-component phosphorelay[59]. Multiple feedback loops influence Spo0A activity, including direct autoregulation of Spo0A. By replacing the P_{spo0A} promoter with a constitutive inducible promoter, Veening et al. showed that the autoregulation of Spo0A is not responsible for bistability of Spo0A activity, nor for epigenetic inheritance[53]. In addition, when they replaced Spo0A with a mutant that phenocopies the phosphorylated form, effectively removing the influence of the phosphorelay, they found that all cells increase expression of Spo0A similarly. Thus, movies enabled the authors to determine which circuit interactions are required for heterogeneous differentiation. These results provide a starting point for further analysis of the role of specific circuit interactions in the heterogeneous process of differentiation.

Coordinating gene expression—A basic problem for cells is how to coordinate the expression of multiple target genes, so that they are expressed in fixed proportions. In bacteria, operons perform this function. How might eukaryotic cells, that lack operons, achieve coordination? In yeast, movies of a fluorescent protein fused to the calcium-responsive transcription factor Crz1p revealed coherent bursts of nuclear localization in which a substantial fraction of Crz1p molecules move together into, and then out of, the nucleus[60]. Strikingly, cells responded to increasing levels of calcium stress by increasing the frequency, but not the duration, of these nuclear localization bursts. Thus, Crz1p activity is regulated by 'frequency modulation' (FM). FM regulation enables Crz1p to coordinate the expression of its many target genes in fixed proportions across many levels of activity. Because the cell regulates the fraction of time Crz1p is active (nuclear localized) rather than fraction of Crz1p molecules in the nucleus, all genes are expressed in proportion to nuclear localization burst frequency. This mechanism coordinates the target genes even when their individual gene regulation functions (GRFs) differ in affinity, cooperativity, etc. Because nuclear localization dynamics are unsynchronized, and appear stochastic, this basic principle of gene regulation could only be identified using movies. It will be interesting to see what role the strategy of FM regulation plays in regulation in other biological systems, from bacteria to multicellular organisms, as similar activity dynamics have been observed in diverse systems [61-64].

Conclusions and future directions

As the examples described above make clear, movies are revealing a whole unexplored world of interesting regulatory strategies, mechanisms, and behaviours. As more systems are imaged in more ways, we anticipate many more interesting discoveries. In model organisms, circuits can be studied more systematically and with greater throughput, analyzing interactions among many pathways or genes simultaneously. Microscopy systems with improved automation can facilitate this transition [65]. Most genetic circuits studied so far involve stress response or differentiation in model organisms, but many other cellular processes, such as metabolism, are amenable as well. It will also be interesting to see whether movies can be used to study circuit behaviours in natural strains or species, in environments that more closely resemble natural conditions. For all of these techniques, an outstanding question is to what extent correlations, even dynamic correlations, can provide sufficient information to infer regulatory interactions or distinguish between possible modes in which circuits may operate.

Synthetic genetic circuits, engineered to implement novel biological functions [42,43] provide unique opportunities to study potential genetic circuit designs. Can such circuits be engineered to operate consistently in all cells? Conversely, can they be designed to mimic biological strategies that take advantage of heterogeneity? Movies provide a powerful means to address these questions.

Some limitations remain. First, despite spectacular work in diversifying the palette of fluorescent protein reporters [66,67], we still have relatively few distinguishable colours that can be used routinely to tag multiple genes. Second, quantitation and tracking of gene expression in individual cells in movie data remains a time-consuming process that is usually optimized differently in each lab. This is often the rate-limiting step for using movies to analyse circuits. More general software tools are needed to expand the usefulness of these techniques. Third, cell–cell interactions remain mysterious. Proximity of cells in movies can provide clues, but the multitude of potential communication mechanisms and channels that exist ensure that many possible interactions can influence cell behaviour. Chemostatic microfluidic systems are beginning to address some of these concerns[68,69]. Similarly, many systems grow in 3-dimensions (for example, biofilms), making quantitation more challenging [70].

Despite these challenges, movies are providing unique insights into how individual cell behaviour results from specific genetic circuit architectures. Only movies can provide a direct view of genetic activities in individual cells — the quantities that we use in mathematical models — that are at best approximated and at worst completely misrepresented by population averages.

The examples described above provide a tantalizing glimpse of the kind of direct insights movies will provide into circuit behaviour. In particular, movie analysis of circuits has already begun to resolve the two seemingly contradictory views of cellular function introduced at the beginning of this review: Variability can be generated from certain circuit designs, such as those incorporating positive feedback. Conversely, circuit functions, such as differentiation, require variability, in the form of stochastic noise.

Box 1

Movie Acquisition and Analysis Techniques

Movie acquisition

A minimal system for time-lapse microscopy consists of an automated fluorescence microscope, software to automate acquisition, and software to assist in tracking and quantification of fluorescence in the resulting movies. The development of microscopes equipped with computer controllable stages, filter wheels and shutters allows for the acquisition of movies of cell growth over several days. Diverse variants of green fluorescent protein [71] now offer three or more distinguishable channels of information in the same cell [66]. Using distinct localization signals can expand this repertoire[67]. Recently, software that automates time-lapse acquisition of images in multiple stage positions is available commercially and as a free open source software package (http://micromanager.org/).

Movie analysis

Analysis of even the most breath-taking movies can require yawn- and/or repetitive strain injury-inducing amounts of time and mouse clicking, respectively. In many systems, the first stage of analysis is segmentation, which identifies the set of pixels belonging to each individual cell on each frame of each movie (Figure 2). Segmentation has been performed successfully on both phase and fluorescent image data using a variety of tactics, including: edge detection, thresholding, and template matching techniques like the Hough transform[72]. Once cells have been identified, tracking algorithms, such as [73], are necessary for inferring cell lineages. Tracking can be a complex problem, as cells divide, grow, rotate, die, or move irregularly. Several software packages are being developed to assist with this problem [74,75], but most are not optimized for bacteria.

Box 2

Issues that impact time-lapse analysis of individual cells

Genetic design considerations

Several issues impact strain design:

- *Reporter type:* Fluorescent protein genes can either be fused to proteins of interest to directly monitor protein levels (protein fusion), or inserted downstream of an additional copy of a natural promoter (promoter fusion). The latter scheme is less likely to disturb the function of the cell, but does not permit analysis of post-translational dynamics, including localization and degradation.

- *Protein stability:* If promoter fusions express stable reporter proteins (GFP and its derivatives are generally stable in bacteria), then it may be difficult to visualize a rapid turn-off in expression, due to lingering previously-expressed proteins. In such cases, one can analyse the rate of protein accumulation (time derivative of fluorescence), rather than the amount of protein [76]. In this case, protein stability can be an advantage, since it removes uncertainty in the degradation rate. On the other hand, time derivatives amplify measurement noise in image data. Another strategy is to destabilize the reporter protein using genetic tags such as those derived from *ssrA* in bacteria [77]. This increases time resolution at the expense of reduced fluorescence levels.
- *Maturation time:* Fluorescent proteins require widely varying times (minutes to hours) to become fluorescent, requiring its direct measurement in the strain of interest. Slow maturation can "smear out" otherwise rapid events in individual cells.

Growth considerations

Growth of cells on a 2D surface permits use of simple, rapid, and light-efficient wide-field fluorescence microscopy. However, cells eventually "pile up", limiting the length of time of observation. Microfluidic chambers have been introduced to maintain chemostatic conditions [25, 69]. In our own work, we find that imaging cells between coverslip and an agarose pad of a few mm provides a relatively simple means of sustaining 2D growth for many cell-generations. While imperfect, this system is sufficient for many applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Marcus JS, Anderson WF, Quake SR. Microfluidic single-cell mRNA isolation and analysis. Anal Chem 2006;78:3084–9. [PubMed: 16642997]
2. Dickinson ME, Bearman G, Tille S, Lansford R, Fraser SE. Multi-spectral imaging and linear unmixing add a whole new dimension to laser scanning fluorescence microscopy. Biotechniques 2001;31:1272, 1274–6, 1278. [PubMed: 11768655]
3. Reddy GV, Heisler MG, Ehrhardt DW, Meyerowitz EM. Real-time lineage analysis reveals oriented cell divisions associated with morphogenesis at the shoot apex of Arabidopsis thaliana. Development 2004;131:4225–37. [PubMed: 15280208]
4. Megason SG, Fraser SE. Imaging in systems biology. Cell 2007;130:784–95. [PubMed: 17803903]
5. Liu AC, et al. Intercellular coupling confers robustness against mutations in the SCN circadian clock network. Cell 2007;129:605–16. [PubMed: 17482552]
6. Maddox P, et al. Microtubule dynamics from mating through the first zygotic division in the budding yeast Saccharomyces cerevisiae. J Cell Biol 1999;144:977–87. [PubMed: 10085295]
7. Straight AF, Sedat JW, Murray AW. Time-lapse microscopy reveals unique roles for kinesins during anaphase in budding yeast. J Cell Biol 1998;143:687–94. [PubMed: 9813090]
8. Raskin DM, de Boer PA. Rapid pole-to-pole oscillation of a protein required for directing division to the middle of Escherichia coli. Proc Natl Acad Sci U S A 1999;96:4971–6. [PubMed: 10220403]
9. Meyer P, Dworkin J. Applications of fluorescence microscopy to single bacterial cells. Res Microbiol 2007;158:187–94. [PubMed: 17349779]
10. Straight AF, Marshall WF, Sedat JW, Murray AW. Mitosis in living budding yeast: anaphase A but no metaphase plate. Science 1997;277:574–8. [PubMed: 9228009]
11. Viollier PH, et al. Rapid and sequential movement of individual chromosomal loci to specific subcellular locations during bacterial DNA replication. Proc Natl Acad Sci U S A 2004;101:9257–62. [PubMed: 15178755]

12. Elowitz MB, Levine AJ, Siggia ED, Swain PS. Stochastic gene expression in a single cell. Science 2002;297:1183–1186. [PubMed: 12183631]
13. Ozbudak EM, Thattai M, Kurtser I, Grossman AD, van Oudenaarden A. Regulation of noise in the expression of a single gene. Nat Genet 2002;31:69–73. [PubMed: 11967532]
14. Raser JM, O'Shea EK. Control of stochasticity in eukaryotic gene expression. Science 2004;304:1811–4. [PubMed: 15166317]
15. Smits WK, Kuipers OP, Veening JW. Phenotypic variation in bacteria: the role of feedback regulation. Nat Rev Microbiol 2006;4:259–71. [PubMed: 16541134]
16. Arkin A, Ross J, McAdams HH. Stochastic kinetic analysis of developmental pathway bifurcation in phage lambda-infected Escherichia coli cells. Genetics 1998;149:1633–48. [PubMed: 9691025]
17. Raser JM, O'Shea EK. Noise in gene expression: origins, consequences, and control. Science 2005;309:2010–3. [PubMed: 16179466]
18. Powell EO. Growth Rate and Generation Time of Bacteria, with Special Reference to Continuous Culture. Journal of General Microbiology 1956;15:492–511. [PubMed: 13385433]
19. Powell EO. An outline of the pattern of bacterial generation times. J Gen Microbiol 1958;18:382–417. [PubMed: 13525657]
20. Wakamoto Y, Inoue I, Moriguchi H, Yasuda K. Analysis of single-cell differences by use of an on-chip microculture system and optical trapping. Fresenius J Anal Chem 2001;371:276–81. [PubMed: 11678202]
21. Kaeberlein M, Burtner CR, Kennedy BK. Recent developments in yeast aging. PLoS Genet 2007;3:e84. [PubMed: 17530929]
22. Stewart EJ, Madden R, Paul G, Taddei F. Aging and death in an organism that reproduces by morphologically symmetric division. PLoS Biol 2005;3:e45. [PubMed: 15685293]
23. Dhar N, McKinney JD. Microbial phenotypic heterogeneity and antibiotic tolerance. Curr Opin Microbiol 2007;10:30–8. [PubMed: 17215163]
24. Lewis K. Programmed death in bacteria. Microbiol Mol Biol Rev 2000;64:503–14. [PubMed: 10974124]
25. Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S. Bacterial persistence as a phenotypic switch. Science 2004;305:1622–5. [PubMed: 15308767]
26. Gefen O, Gabay C, Mumcuoglu M, Engel G, Balaban NQ. Single-cell protein induction dynamics reveals a period of vulnerability to antibiotics in persister bacteria. Proc Natl Acad Sci U S A 2008;105:6145–9. [PubMed: 18427112]
27. McKinney JD. In vivo veritas: the search for TB drug targets goes live. Nat Med 2000;6:1330–3. [PubMed: 11100116]
28. Moyed HS, Bertrand KP. hipA, a newly recognized gene of Escherichia coli K-12 that affects frequency of persistence after inhibition of murein synthesis. J Bacteriol 1983;155:768–75. [PubMed: 6348026]
29. Kaufmann BB, Yang Q, Mettetal JT, van Oudenaarden A. Heritable stochastic switching revealed by single-cell genealogy. PLoS Biol 2007;5:e239. [PubMed: 17803359]
30. Di Talia S, Skotheim JM, Bean JM, Siggia ED, Cross FR. The effects of molecular noise and size control on variability in the budding yeast cell cycle. Nature 2007;448:947–51. [PubMed: 17713537]
31. Bean JM, Siggia ED, Cross FR. Coherence and timing of cell cycle start examined at single-cell resolution. Mol Cell 2006;21:3–14. [PubMed: 16387649]
32. Nachman I, Regev A, Ramanathan S. Dissecting timing variability in yeast meiosis. Cell 2007;131:544–56. [PubMed: 17981121]
33. Tomita J, Nakajima M, Kondo T, Iwasaki H. No transcription-translation feedback in circadian rhythm of KaiC phosphorylation. Science 2005;307:251–4. [PubMed: 15550625]
34. Nakajima M, et al. Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro. Science 2005;308:414–5. [PubMed: 15831759]
35. Elowitz MB, Leibler S. A synthetic oscillatory network of transcriptional regulators. Nature 2000;403:335–338. [PubMed: 10659856]
36. Mihalcescu I, Hsing W, Leibler S. Resilient circadian oscillator revealed in individual cyanobacteria. Nature 2004;430:81–5. [PubMed: 15229601]

37. Chabot JR, Pedraza JM, Luitel P, van Oudenaarden A. Stochastic gene expression out-of-steady-state in the cyanobacterial circadian clock. Nature 2007;450:1249–52. [PubMed: 18097413]
38. Amdaoud M, Vallade M, Weiss-Schaber C, Mihalcescu I. Cyanobacterial clock, a stable phase oscillator with negligible intercellular coupling. Proc Natl Acad Sci U S A 2007;104:7051–6. [PubMed: 17438272]
39. Garcia-Ojalvo J, Elowitz MB, Strogatz SH. Modeling a synthetic multicellular clock: repressilators coupled by quorum sensing. Proc Natl Acad Sci U S A 2004;101:10955–60. [PubMed: 15256602]
40. Rust MJ, Markson JS, Lane WS, Fisher DS, O'Shea EK. Ordered phosphorylation governs oscillation of a three-protein circadian clock. Science 2007;318:809–12. [PubMed: 17916691]
41. Rosenfeld N, Young JW, Alon U, Swain PS, Elowitz MB. Accurate prediction of gene feedback circuit behavior from component properties. Mol Syst Biol 2007;3:143. [PubMed: 18004276]
42. Endy D. Foundations for engineering biology. Nature 2005;438:449–53. [PubMed: 16306983]
43. Sprinzak D, Elowitz MB. Reconstruction of genetic circuits. Nature 2005;438:443–8. [PubMed: 16306982]
44. Andrianantoandro E, Basu S, Karig DK, Weiss R. Synthetic biology: new engineering rules for an emerging discipline. Mol Syst Biol 2006;2 2006 0028.
45. Grilly C, Stricker J, Pang WL, Bennett MR, Hasty J. A synthetic gene network for tuning protein degradation in Saccharomyces cerevisiae. Mol Syst Biol 2007;3:127. [PubMed: 17667949]
46. Yu J, Xiao J, Ren X, Lao K, Xie XS. Probing gene expression in live cells, one protein molecule at a time. Science 2006;311:1600–3. [PubMed: 16543458]
47. Cai L, Friedman N, Xie XS. Stochastic protein expression in individual cells at the single molecule level. Nature 2006;440:358–62. [PubMed: 16541077]
48. Friedman N, Cai L, Xie XS. Linking stochastic dynamics to population distribution: an analytical framework of gene expression. Phys Rev Lett 2006;97:168302. [PubMed: 17155441]
49. Dubnau D. Genetic competence in Bacillus subtilis. Microbiol Rev 1991;55:395–424. [PubMed: 1943994]
50. Maamar H, Dubnau D. Bistability in the Bacillus subtilis K-state (competence) system requires a positive feedback loop. Mol Microbiol 2005;56:615–24. [PubMed: 15819619]
51. Suel GM, Garcia-Ojalvo J, Liberman LM, Elowitz MB. An excitable gene regulatory circuit induces transient cellular differentiation. Nature 2006;440:545–550. [PubMed: 16554821]
52. Suel GM, Kulkarni RP, Dworkin J, Garcia-Ojalvo J, Elowitz MB. Tunability and noise dependence in differentiation dynamics. Science 2007;315:1716–1719. [PubMed: 17379809]
53. Veening JW, et al. Bet-hedging and epigenetic inheritance in bacterial cell development. Proc Natl Acad Sci U S A 2008;105:4393–8. [PubMed: 18326026]
54. Hamoen LW, Venema G, Kuipers OP. Controlling competence in Bacillus subtilis: shared use of regulators. Microbiology 2003;149:9–17. [PubMed: 12576575]
55. Keener, J.; Sneyd, J. Mathematical Physiology. Springer; New York: 1998.
56. Maamar H, Raj A, Dubnau D. Noise in gene expression determines cell fate in Bacillus subtilis. Science 2007;317:526–9. [PubMed: 17569828]
57. Piggot PJ, Hilbert DW. Sporulation of Bacillus subtilis. Curr Opin Microbiol 2004;7:579–86. [PubMed: 15556029]
58. Veening JW, Hamoen LW, Kuipers OP. Phosphatases modulate the bistable sporulation gene expression pattern in Bacillus subtilis. Mol Microbiol 2005;56:1481–94. [PubMed: 15916600]
59. Jiang M, Shao W, Perego M, Hoch JA. Multiple histidine kinases regulate entry into stationary phase and sporulation in Bacillus subtilis. Mol Microbiol 2000;38:535–42. [PubMed: 11069677]
60. Cai L, Dalal C, Elowitz MB. Nature. 2008 In Press.
61. Nelson DE, et al. Oscillations in NF-kappaB signaling control the dynamics of gene expression. Science 2004;306:704–8. [PubMed: 15499023]
62. Lahav G, et al. Dynamics of the p53-Mdm2 feedback loop in individual cells. Nat Genet 2004;36:147–50. [PubMed: 14730303]
63. Garmendia-Torres C, Goldbeter A, Jacquet M. Nucleocytoplasmic oscillations of the yeast transcription factor Msn2: evidence for periodic PKA activation. Curr Biol 2007;17:1044–9. [PubMed: 17570669]

64. Medvedik O, Lamming DW, Kim KD, Sinclair DA. MSN2 and MSN4 link calorie restriction and TOR to sirtuin-mediated lifespan extension in Saccharomyces cerevisiae. PLoS Biol 2007;5:e261. [PubMed: 17914901]

65. Pepperkok R, Ellenberg J. High-throughput fluorescence microscopy for systems biology. Nat Rev Mol Cell Biol 2006;7:690–6. [PubMed: 16850035]

66. Shaner NC, Steinbach PA, Tsien RY. A guide to choosing fluorescent proteins. Nat Methods 2005;2:905–9. [PubMed: 16299475]

67. Livet J, et al. Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. Nature 2007;450:56–62. [PubMed: 17972876]

68. Groisman A, et al. A microfluidic chemostat for experiments with bacterial and yeast cells. Nat Methods 2005;2:685–9. [PubMed: 16118639]

69. Cookson S, Ostroff N, Pang WL, Volfson D, Hasty J. Monitoring dynamics of single-cell gene expression over multiple cell cycles. Mol Syst Biol 2005;1 2005 0024.

70. Vlamakis H, Aguilar C, Losick R, Kolter R. Control of cell fate by the formation of an architecturally complex bacterial community. Genes Dev 2008;22:945–53. [PubMed: 18381896]

71. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. Science 1994;263:802–5. [PubMed: 8303295]

72. Gonzalez, RC.; Woods, RE. Digital Image Processing. second edition. 2002.

73. Victoria, G.; Michael, E.; Tigran, B.; Eric, M. Tracking Cell Signals in Fluorescent Images. Proceedings of the 2005 IEEE Computer Society Conference on Computer Vision and Pattern Recognition (CVPR'05) - Workshops - Volume 03; IEEE Computer Society; 2005.

74. Gordon A, et al. Single-cell quantification of molecules and rates using open-source microscope-based cytometry. Nat Methods 2007;4:175–81. [PubMed: 17237792]

75. Shen H, et al. Automated tracking of gene expression in individual cells and cell compartments. J R Soc Interface 2006;3:787–94. [PubMed: 17015304]

76. Friedman N, Vardi S, Ronen M, Alon U, Stavans J. Precise temporal modulation in the response of the SOS DNA repair network in individual bacteria. PLoS Biol 2005;3:e238. [PubMed: 15954802]

77. Andersen JB, et al. New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. Appl Environ Microbiol 1998;64:2240–6. [PubMed: 9603842]

78. Rosenfeld N, Young JW, Alon U, Swain PS, Elowitz MB. Gene regulation at the single-cell level. Science 2005;307:1962–1965. [PubMed: 15790856]

Glossary

Linear micro-fluidic chambers	Microfluidic devices in which cells are confined to grow in a narrow groove. These devices facilitate analysis of cell lineages, since more closely related cells are located closer together.
Noise	Fluctuations in molecular components. Noise arises due to the low copy numbers of molecular species and the burst-like nature of transcription, among other mechanisms.
Galactose utilization system	A system of genes used by yeast to control the uptake and metabolism of galactose. This system is characterized by several feedback loops, both positive and negative.
Repressilator	A synthetic genetic circuit designed to produce clock-like oscillations in the levels of is components. The circuit consists of a ‘rock-scissors-paper’ feedback loop of three repressors, in which the first represses the expression of the second, the second the third, and the third the first.
Spo0A	Master transcriptional regulator for sporulation in *B.subtilis*. Spo0A is controlled by phosphorylation and transcriptional regulation.
Segmentation	breaking up a complex image into individual objects, such as cells.

Edge detection	A computational algorithm that identifies sharp changes in intensity, associated with boundaries between objects, such as cells.
Thresholding	One of the simplest segmentation techniques based on identifying groups of pixels whose intensity exceeds a defined cutoff value.
Hough transform	An algorithm for identifying particular shapes, such as circular disks, in complex images. The Hough transform is useful in many segmentation systems.

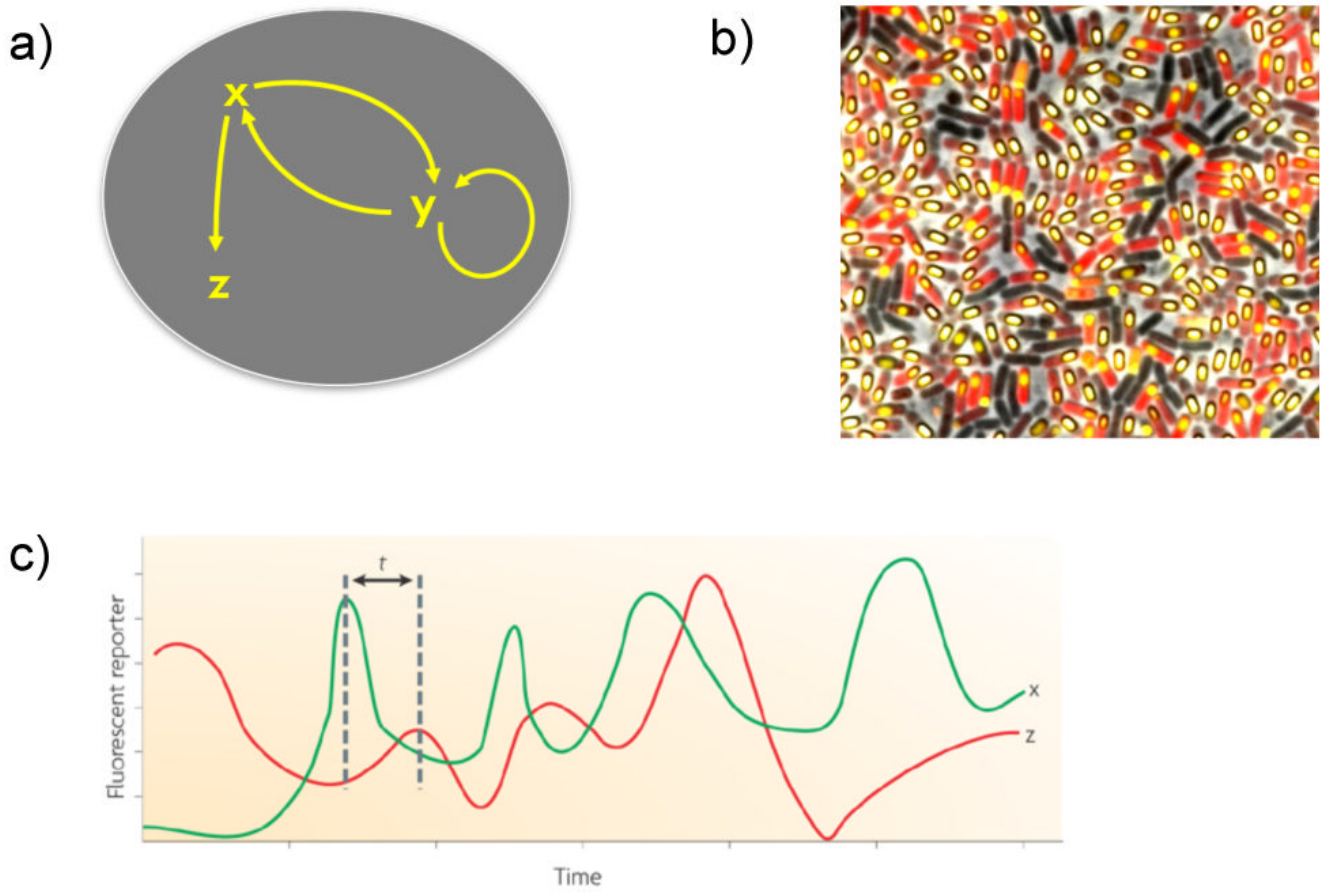

Figure 1. Circuits, variability, and movies

A) Circuit level view: genes and gene products interact to generate an ordered behavioural program. B) Noisy view: Isogenic populations exhibit large degrees of heterogeneity, both in terms of gene expression and differentiated states. As an example, we show an image of a *B. subtilis* strain with two chromosomally integrated reporter constructs, P_{spoIIq}-*cfp* (shown in yellow), P_{spoIID}-*yfp* (shown in red), superimposed on a phase contrast image (gray). Cells were grown in sporulation medium. However, they initiate sporulation at different times, leading vegetative cells (dark rods) to coexist with cells various stages of sporulation (coloured cells). C) Movies allow us to analyse the effects of circuit interactions on the relative timing of gene expression in variable and dynamic circuits. Here, two schematic gene expression traces are shown in red and green for a simple activating interaction (x activates z). Note that the movie enables one to observe delayed correlations that would not be evident in snapshots. τ indicates a typical delay before regulatory effects of x are visible in z.

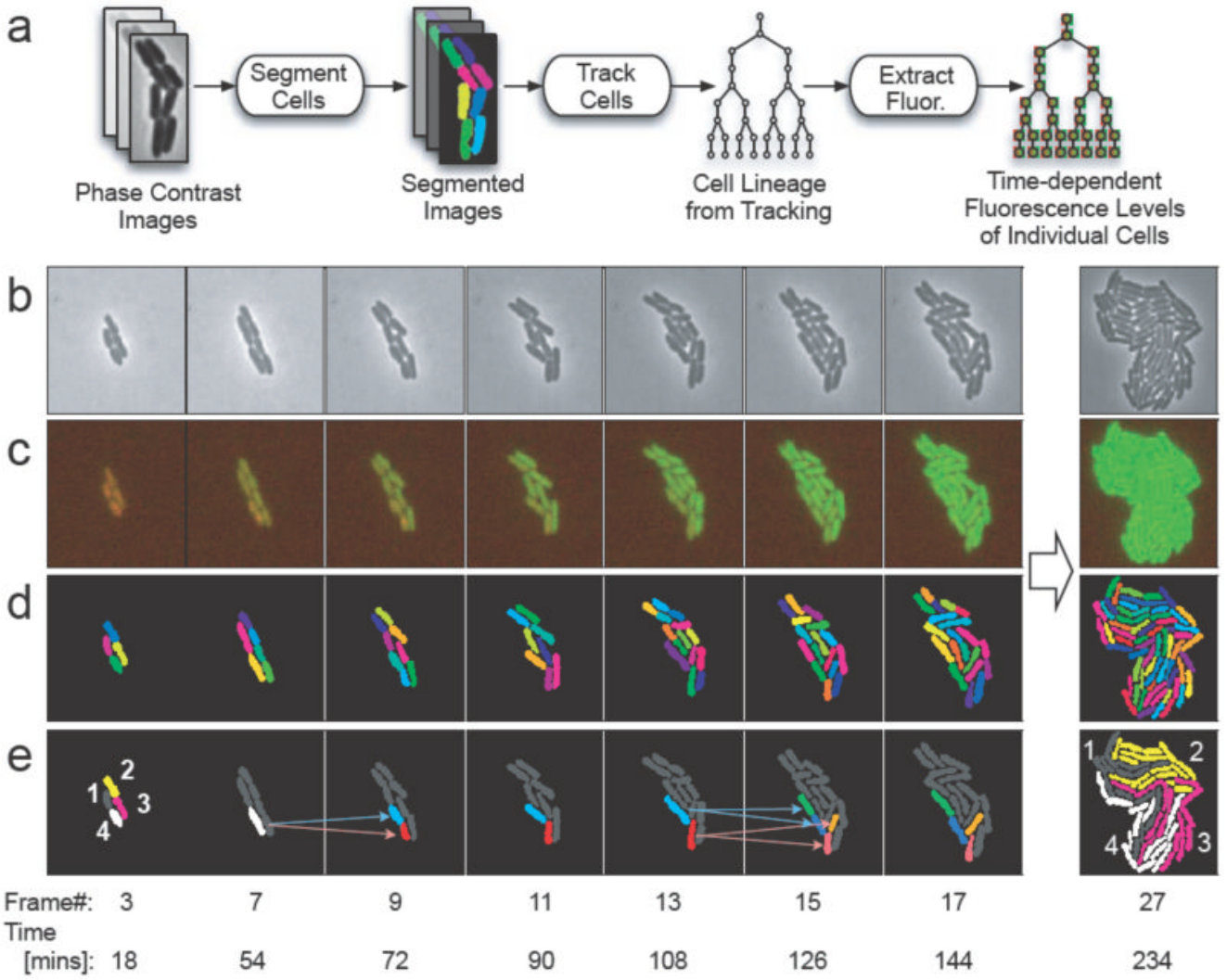

Figure 2. Tracking and segmenting single cells

A) Schematic of data flow for a cell tracking and segmentation system. During tracking cell shapes must first be identified in images (segmentation) and then tracked over time. Finally the fluorescence values must be extracted. B-E) Segmentation and tracking input and output: B) Phase-contrast images are obtained at time-intervals (shown at the bottom). C) Fluorescence images of the microcolony. In this example, filters for yellow and cyan fluorescent proteins are used (shown in red and green respectively). D) Segmentation, performed on the phase-contrast images, finds the locations of each cell in the image. Arbitrary colours are used for labelling. E) The descendents of cell #4, are shown highlighted. The final panel shows the descendants of each of the 4 initial cells after ≈ 4 generations. Figure is courtesy of J. Young, California Institute of Technology, California, USA, and N. Rosenfeld, Rosetta Genomics, Rehovot, Israel.

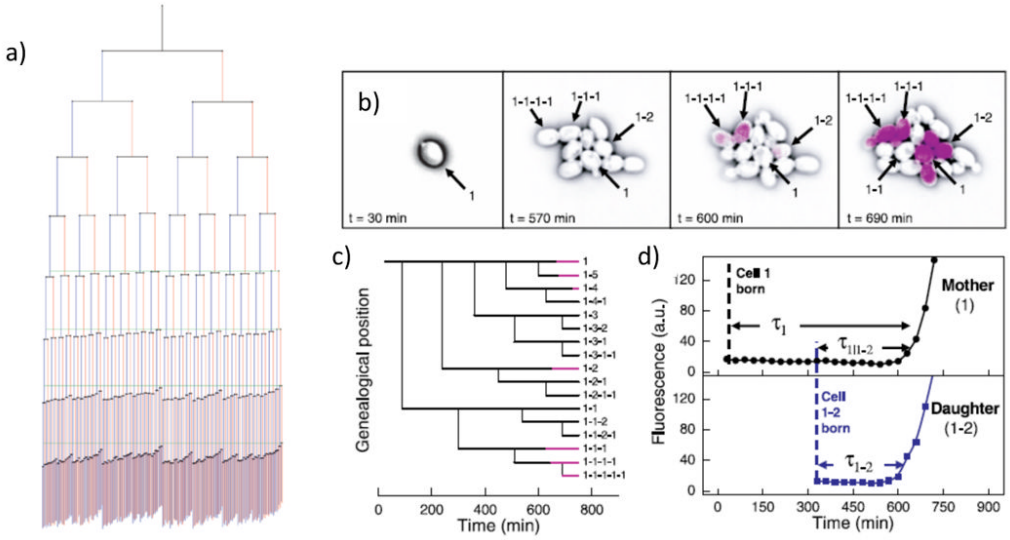

Figure 3. Automated lineage analysis reveals epigenetic states

A) The aging of *Escherichia coli* (from [22]). This lineage tree depicts 9 generations of E. coli from 94 movies. The lengths of the lines connecting cells to their progeny are proportional to the average growth rate of that cell, so a shorter line represents a shorter growth rate. At each division, the cell inheriting the old pole is placed on the right side of the division pair, and shown in red, while new poles are placed on the left side of each pair, and shown in blue. Green lines indicate the point at which the first cell divides in the last four generations. B-D) Genealogical switching history in the Yeast Galactose system (from [29]). The first cell in each movie is designated cell number 1 and sequential daughters of that cell 1–1, 1–2, 1–3. These daughter cells bud in turn, giving rise to cells 1-1-1, 1-1-2, 1-2-1, etc. (B) An initially OFF cell grows into a variegated microcolony. Beginning at 600 min, or 4 generations, several cells fluoresce almost simultaneously. This includes the mother-daughter pairs (1,1–2) and (1-1-1,1-1-1–1). Conspicuously, cell 1–1 does not switch for the duration of the movie, even though its mother, daughter, and closest sibling all do. (C) The family tree for colony in (B). Black lines indicate cells in the OFF state, whereas pink lines represent cells after they have switched to the ON state.(D) Fluorescent time courses for mother cell 1 and her daughter 1–2, showing each as they switch into the ON state.

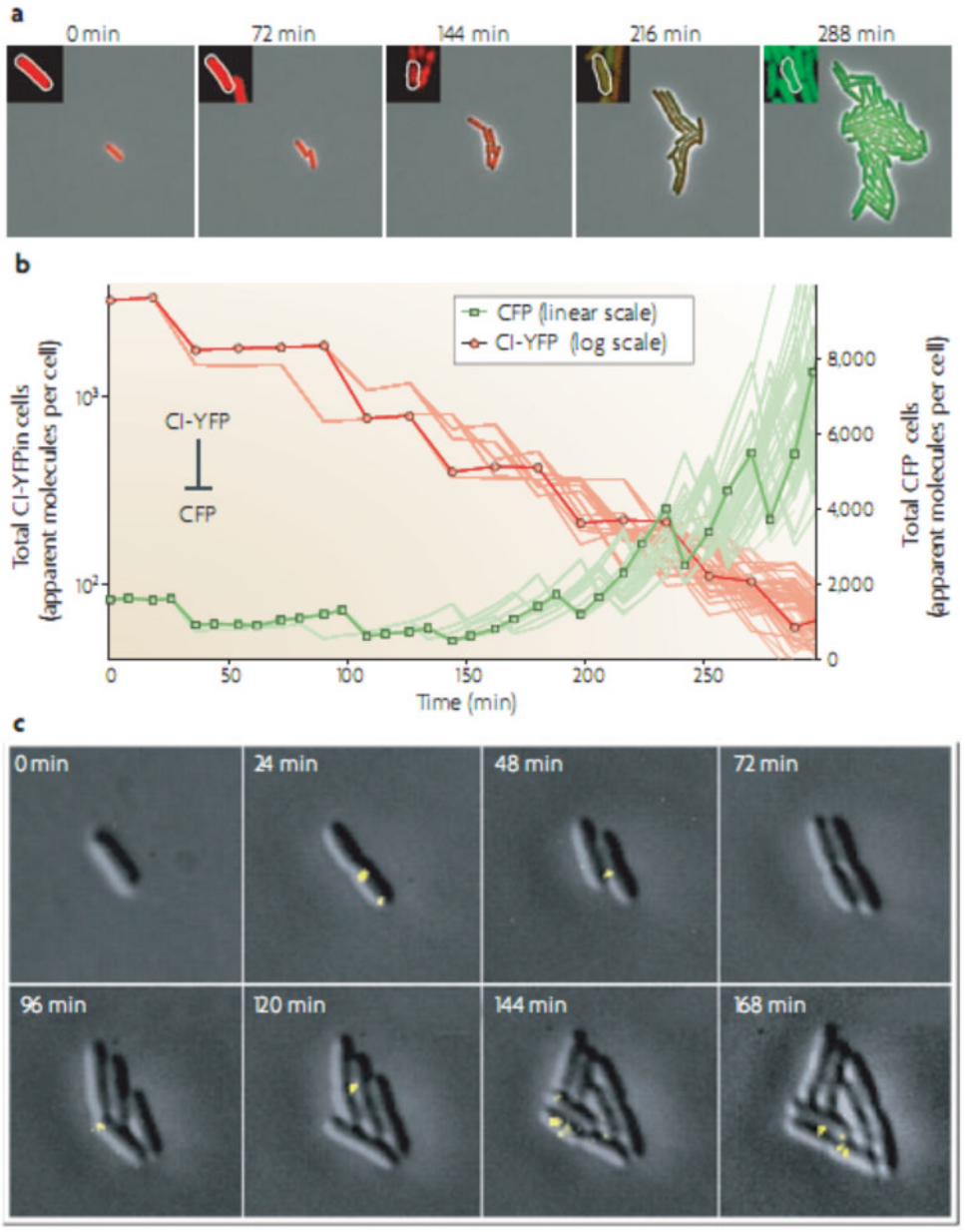

Figure 4. *In vivo* Biochemistry

A-B) Measuring the gene regulation function (GRF) of a repressor-promoter interaction in individual *E. coli* cell lineages (from [78]). Here, CI-YFP (lambda repressor fused to yellow fluorescent protein) represses expression of cyan fluorescent protein (CFP). In the regulator dilution experiment: Cells are transiently induced to express CI-YFP and then observed in time-lapse microscopy as repressor dilutes out during cell growth. Part A) shows a filmstrip of a typical experiment. CI-YFP is shown in red and CFP is shown in green. Part B) shows quantitation of the movie. CI-YFP levels decrease by dilution (red lines), eventually permitting expression of the *cfp* target gene (green lines). The darker lines correspond to the cell lineage shown in the insets to part A). C) Monitoring transcriptional bursts in single cells (from [46]). Frames from film footage of the expression of Tsr-Venus under the control of a repressed lac promoter. Tsr-Venus expression is shown in yellow and is overlaid with simultaneous DIC images (differential interference contrast) images (gray). Note the burst like expression pattern.

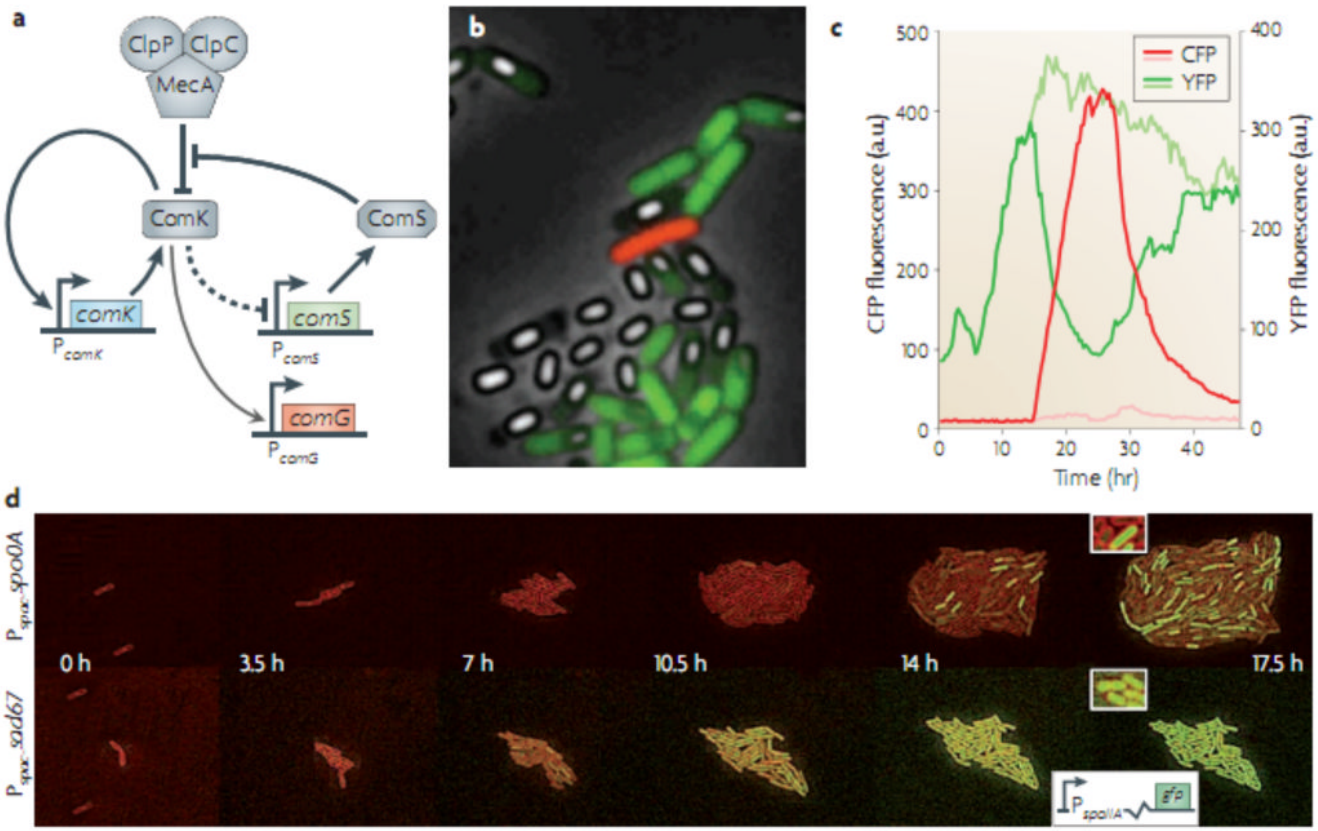

Figure 5. Circuit level Dynamics

A-C) Analysis of Bacillus subtilis competence circuit dynamics in individual cells (from[51]). Part A) shows a snapshot from a movie. P_{comS} expression is shown in green and P_{comG} expression is shown in red. The red cell is in the competent state (high ComK levels). White depicts spores or sporulating cells. Part b shows a quantitative time series of P_{comS} –yellow fluorescent protein (*yfp*) (green lines) and P_{comG} –cyan fluorescent protein (cfp) red lines) for the competence event shown in A). Note the anti-correlation in expression between the two promoters, which can be explained by the circuit diagram in part C. P_{comS} and P_{comG} activities obtained from the non-competent sister cell are shown in light green and light red, respectively. Part C) shows a map of the effective regulatory interactions in the core competence circuit in *B. subtilis*. The dashed inhibitory arrow depicts indirect repression. ComS competes with ComK for degradation by the MecA–ClpP–ClpC complex, effectively stabilizing ComK. D) The *B. subtilis* phosphorelay is required to generate variability in sporulation in *B. subtilis* (From [53]). Time-lapse microscopy shows that heterogeneity in this system does not require the positive-feedback loop of Spo0A on itself (top row), but does require the activity of the phosphorelay (bottom row). Membranes are stained with FM5–95 (red), and expression of the sporulation reporter P_{spoIIA} is shown in green. The insets show a close-up of the cells.